THIS PAGE BLANK (USPTO)

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6]: **C12N 15/09, 15/12, C07K 14/74**	**A1**	(11) International Publication Number: **WO 99/11775** (43) International Publication Date: 11 March 1999 (11.03.99)

(21) International Application Number: PCT/US98/17308

(22) International Filing Date: 20 August 1998 (20.08.98)

(30) Priority Data:
08/920,413   29 August 1997 (29.08.97)   US

(63) Related by Continuation (CON) or Continuation-in-Part
(CIP) to Earlier Application
US   08/920,413 (CON)
Filed on   29 August 1997 (29.08.97)

(71) Applicants *(for all designated States except US)*: PRESIDENT
AND FELLOWS OF HARVARD COLLEGE [US/US]; 17
Quincy Street, Cambridge, MA 02138 (US). THE GEN-
ERAL HOSPITAL CORPORATION [US/US]; 55 Fruit
Street, Boston, MA 02114 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WALKER, Jürgen, B.
[DE/US]; Hirschensprung 1, D–91056 Erlangen (DE).
GARBOCZI, David, N. [US/US]; 17613 Parkridge Drive,
Gaithersburg, MD 20878 (US).

(74) Agent: TSAO, Y., Rocky; Fish & Richardson P.C., 225
Franklin Street, Boston, MA 02110–2804 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH,
CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL,
PT, SE).

Published
With international search report.

(54) Title: MUTANT MHC CLASS I MOLECULES

(57) Abstract

Methods of generating a conjugate of MHC class I molecule and a compound via a cysteine residue engineered into the $\beta 2$–M subunit. Also featured are uses of the new conjugates.

MUTANT MHC CLASS I MOLECULES

Statement As to Federally Sponsored Research

Background of the Invention

The major histocompatibility complex ("MHC") plays
10 a central role in the immune system. Antigen-specific T
cells recognize antigenic peptides in association with
MHC class I or II molecules on the cell surface. Class I
molecules consist of two noncovalently associated
subunits: a highly polymorphic α heavy chain and a
15 conserved $\beta2$-microglobulin ("$\beta2$-M") light chain. Two of
the three extracellular domains of the heavy chain, i.e.,
domains $\alpha1$ and $\alpha2$, are folded into a "groove" structure
which anchors an antigenic peptide for presentation to T
cells.

20   Human class I molecules (or "complexes") have been
refolded from *E. coli*-produced heavy chains and $\beta2$-M
subunits in the presence of synthetic peptides (Garboczi
et al., *Proc. Natl. Acad. Sci. USA*, 89:3429-3433, 1992).
The three-dimensional structures of such recombinant
25 complexes as determined by X-ray crystallography are
virtually identical to the structure of the class I
molecule as isolated from human cells (Madden et al.,
Cell, 75:693-708, 1993; Bjorkman et al., *Nature*, 329:506-
512, 1987). Further, subtype A0201* of HLA-A2 produced
30 in *E. coli* and assembled with synthetic HIV-1
nonapeptides has been shown to elicit cytolytic CD8^{+} T
cell responses (Walter et al., *Int. Immunology*, 9:451-
459, 1997).

- 2 -

The classical class I gene family includes the highly polymorphic human class I molecules HLA-A, -B, and -C, and murine class I (i.e., H-2) molecules D, K, and L. A series of structural relatives (non-classical class I molecules) has been found in humans (e.g., HLA-E, -F, -G, -H, -I, and -J; and CD1) and mice (Q, T, M, and CD1) (Shawar et al., *Annu. Rev. Immunol.*, 12:839-880, 1994). These molecules have the typical structure of an antigen-presenting molecule, where a polymorphic heavy chain is noncovalently associated with the conserved β2-M subunit. The T cell repertoire reacting with these non-classical ligands has been characterized to only a limited extent.

Summary of the Invention

The invention features a method of preparing a conjugate of an MHC class I molecule and a compound. In this method, one first obtains an MHC class I molecule, where a cysteine residue (i.e. a non-natural or new cysteine residue) has been engineered into its β2-microglobulin subunit. The compound (e.g., a protein, a carbohydrate, a lipid molecule, or any other organic compound) is then conjugated to the mutant class I molecule specifically via a linkage formed between the sulfhydryl group of the new cysteine residue in the β2-microglobulin subunit and a functional group of the compound. Alternatively, the compound can be first conjugated to the new β2-M subunit, and then the subunit is mixed with an α heavy chain (from the same or different species as the β2-M subunit) in the presence of an appropriate peptide to form a compound-class I conjugate. The cysteine residue is preferably introduced into a region of β2-M that faces away from the interface between β2-M and the α heavy chain. Exemplary regions are those corresponding to residues 15-23, 35-53, or 66-97 of SEQ ID NO:1. SEQ ID NO:1 shows the amino acid

sequence of a human β2-M. Minor sequence variations can
exist among β2-M molecules from different or the same
species; and residues from two different β2-M sequences
are said to be corresponding to each other when they are
5 equivalent in function or relative position to the
conserved residues in the two β2-M sequences, or both.
The new residue, alone or together with one or more
(e.g., two to five) amino acid residues, can be inserted
into the β2-M region without any deletion of the region,
10 or replace one or more (e.g., two to five) residues of
the region. For instance, the new cysteine residue can
replace a residue that corresponds to serine 52, tyrosine
67, or lysine 91 of SEQ ID NO:1.
        The compound can be, for example, a ligand for a
15 multivalent binding molecule, an antibody (e.g., one that
is specific for a tumor antigen), a molecule on the
surface of a cell (e.g., an antigen-presenting cell or
any other hematopoietic cell), or a ligand for a surface
receptor of a cell.
20   The new compound-class I conjugates have several
uses. For instance, when a multivalent binding molecule
is supplied, conjugates of the ligands for the binding
molecule with the new, monomeric class I molecules (e.g.,
ones that consist of an α heavy chain, the new β2-M, and
25 a peptide associated with the heavy chain) can be
multimerized. Multimeric class I molecules can be used
to, e.g., label, isolate and quantitate specific T cells.
Exemplary multivalent binding molecules are avidin (or a
derivative thereof, e.g., streptavidin), whose ligands
30 are biotin and biotin derivatives.
        A conjugate of the present invention can also be
used to stimulate the immunity of an individual (e.g., a
human or a mouse). To accomplish this, a conjugate of a
new class I molecule and a cell (e.g., an antigen-
35 presenting cell) is introduced into an individual, and

- 4 -

the conjugate can stimulate the immune cells,
particularly T cells specific for the peptide in the
conjugate. In this method, the cell and the class I
molecule in the conjugate are preferably syngeneic with
5 this individual.

    Another conjugate of the present invention can be
used to eradicate a tumor (or any other undesired cell)
in an individual. In this method, a conjugate of a new
class I molecule and an antibody specific for an antigen
10 (or a ligand for a receptor) expressed exclusively or
primarily on the cell is introduced into the individual.
The α heavy chain in the conjugate can be allogeneic or
xenogeneic) to the patient. If the heavy chain is
syngeneic to the patient, the class I molecule would be
15 associated with an antigenic peptide that can elicit a
strong T cell response.

    Use of the new recombinant $\beta2$-M, monomeric and
multimeric class I molecules containing the new $\beta2$-M, and
the new compound-class I conjugates are also within the
20 scope of the invention.

    The new $\beta2$-M eliminates the need for genetically
engineering MHC heavy chains to create a chemically
reactive site in each of the differing MHC class I
molecules. This is a significant advantage in view of
25 the enormous polymorphism of MHC heavy chains.

    Other features and advantages of the invention
will be apparent from the following detailed description,
and from the claims.

                    Brief Description of the Drawings
30   Figs. 1A-1C are a set of graphs showing the
results of flow cytometry analysis of the binding of the
phycoerythrin-labeled HLA-A2-SL9 tetramer to human
cytotoxic T lymphocyte cells of clones 68A62 (specific
for HLA-A2-IV9), 18030 (specific for HLA-A2-SL9), and

- 5 -

63D35 (specific for HLA-B11-AK9), respectively. The
cells were also stained with an anti-CD8 monoclonal
antibody that was labeled with Cy Chrome, i.e., a
fluorescent complex of phycoerythrin and Texas Red.

5   Figs. 2A-2C are a set of graphs showing the
results of flow cytometry analysis of the binding of the
phycoerythrin-labeled chimeric $H-2K^b$-SV9 tetramer to mouse
cytotoxic T lymphocytes of clones 2F3 (specific for the
SV9 peptide bound to $H-2K^b$), 3C2 (specific for the SV9
10 peptide bound to $H-2D^b$), and 4G3 (specific for the OVA
peptide bound to $H-2K^b$), respectively. The staining
intensity of the $H-2K^b$-SV9 tetramer is shown in solid
line, and the intensity of background fluorescence (i.e.,
staining with avidin-PE only) is shown in dashed line.

15   Figs. 3A and 3B are a set of graphs showing the
binding of a tetrameric MHC-peptide complex ($H-2K^b$
complexed with SV9) to CTLs where K^b is syngeneic (Fig.
3A) or allogeneic (Fig. 3B). Dual parameter contour
plots in Figs. 3A and 3C illustrate the phenotype (anti-
20 CD8 *versus* anti-$H-2K^b$) of CTLs (double positive) generated
in $H-2^b$ mice and CTLs (single positive) generated in $H-2^d$
mice, respectively. The staining intensities with the H-
$2K^b$ SV9 reagent (bold line) in Figs. 3A and 3C are shown
in Figs. 3B and 3D, respectively, in comparison with
25 background intensity (avidin-PE only, dashed line). Note
the comparable shift of fluorescence intensity for
syngeneic (Fig. 3B) and the majority of the allogeneic
CTL (Fig. 3D).

Detailed Description

30   Methods are provided for preparing a conjugate of
a MHC class I molecule and a compound. The MHC class I
molecule contains a recombinant $\beta 2$-M subunit in which a
cysteine residue has been introduced. This residue is
preferably introduced into a region of $\beta 2$-M that does not

- 6 -

interact with the α heavy chain. Standard mutagenesis
techniques can be employed to generate DNA encoding such
mutant β2-M molecules. General guidance can be found in
Sambrook et al., *Molecular Cloning, A Laboratory Manual*,
5 Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
New York, 1989; and Ausubel et al., *Current Protocols in
Molecular Biology*, Greene Publishing and Wiley-
Interscience, New York, New York, 1993. The cysteine
residue introduced into the β2-M subunit provides a
10 convenient site for highly selective chemical
modification. In addition, coupling between sulfhydryls
and certain functional groups is reversible, allowing a
compound to be released from the class I molecule to
which it has been conjugated.

15   The compound can be linked to the new cysteine
residue in the class I molecule via a functional group of
its own. Sulfhydryl-reactive functional groups include,
but are not limited to, maleimides, pyridyl disulfide,
α-haloacyl derivatives (e.g., iodoacetamides), alkyl
20 halides, and aryl halides. Maleimides, alkyl and aryl
halides, and α-haloacyls react with sulfhydryls to form
thiol ether bonds. Pyridyl disulfides, on the other
hand, react with sulfhydryls to produce mixed disulfides.
The pyridyl disulfide product is cleavable. A
25 sulfhydryl-reactive crosslinker can also be used,
especially when the compound does not contain any
sulfhydryl-reactive groups itself. Such a crosslinker
possesses two or more different reactive groups that
allow its sequential conjugations with two or more other
30 molecules. For instance, crosslinkers that are amine-
reactive at one part and sulfhydryl-reactive at another
part (e.g., some DOUBLE-AGENT™ crosslinkers available
from PIERCE, Rockford, Illinois) can be used to link an
amine-containing compound with the mutant class I
35 molecule. By way of example, N-Succinimidyl

3-(2-pyridyldithio)-propionate ("SPDP") is a reversible
NHS-ester (i.e., N-hydroxysuccinamide-ester), pyridyl
disulfide cross-linker; the amine-reactive NHS-ester in
SPDP can be reacted first with a compound of interest,
5   and then with the free -SH group of the new class I
molecule. Useful water-soluble SPDP analogs include
sulfosuccinimidyl 6-(3-[2-pyridyldithio]propionamido)
hexanoate (i.e., sulfonating long chain SPDP analog or
Sulfo-LC-SPDP), LC-SPDP, 4-Succinimidyloxycarbonyl-
10   α-methyl-α-(2-pyridyldithio)-toluene ("SMPT"), and
sulfosuccinimidyl-6-(α-methyl-α-[2-pyridyldithio]-
toluamido) hexamoate ("Sulfo-LC-SMPT"). Additional
sulfhydryl- and amine-reactive heterofunctional
crosslinkers include succinimidyl-4-(N-maleimidomethyl)-
15   cyclohexane-1-carboxylate ("SMCC"), N-γ-
maleimidobutyryloxysulfosuccinimide ester, m-
maleimidobenzoyl-N-hydroxysulfosuccinimide ester,
sulfosuccinimidyl [4-iodoacetyl] aminobenzoate, and
sulfosuccinimidyl 4-[p-maleimidophenyl] butyrate (Pierce,
20   Illinois). Homobifunctional sulfhydryl-reactive
crosslinkers such as Bis-maleimidohexane ("BMH"), 1,4-Di-
(3'-[2'-pyridyldithio] propionamido-butane ("DPDPB"), and
1,5-difluoro-2,4-dinitrobenzene ("DFDNB") can also be
used if the compound to be conjugated contains a
25   sulfhydryl.

        The α and β2-M subunits of the MHC class I protein
can be from the same or different species (e.g., humans,
rats, mice, hamsters, frogs, chickens, etc.). The
transmembrane and optionally intracellular domains of the
30   α subunit can be removed to promote proper *in vitro*
folding. Methods for obtaining class I heavy chains and
β2-M subunits and for forming monomeric class I-peptide
complexes are well known in the art (see, e.g., Zhang et
al., *Proc. Natl. Acad. Sci. USA*, 89:8403-8407, 1992; and
35   Garboczi et al., *Proc. Natl. Acad. Sci. USA*, 89:3429-

- 8 -

3433, 1992). The α and β2-M subunits obtained with a
recombinant expression system (e.g., an *E. coli* or
baculoviral system) can be refolded separately or
together, and then associated in the presence of
5   appropriate peptides (e.g., peptides of about 8-12 amino
acid residues in length). Alternatively, the mutant β2-M
subunit can be associated with the α subunit of a pre-
formed class I molecule via exchange with the β2-M
subunit in the pre-formed molecules; in such case, no
10 peptide supply is needed in the association reaction if
the pre-formed class I molecule is already occupied by a
peptide.

    In general, the conjugation between the class I
protein and the compound is performed after the β2-M
15 subunit has folded into a native or native-like
conformation. In this conformation, the naturally
occurring cysteine residues (4 in the heavy chain and 2
in β2-M) are engaged in stable disulfide bonds and hence
not accessible for chemical modification. Therefore,
20 conjugation occurs specifically via the unpaired,
surface-exposed cysteine.

    Described below are the generation and use of
several conjugates of the invention. Unless otherwise
defined, all technical and scientific terms used herein
25 have the same meaning as commonly understood by one of
ordinary skill in the art to which this invention
belongs. Although methods and materials similar or
equivalent to those described herein can be used in the
practice or testing of the present invention, exemplary
30 methods and materials are described below. These
exemplary methods and materials are illustrative only and
not intended to be limiting. All publications, patent
applications, patents, and other references mentioned
herein are incorporated by reference in their entirety.

In case of conflict, the present specification, including definitions, will control.

I.   Cell-Class I Conjugates

The new class I molecule can be conjugated to a
5 compound (e.g., a protein, carbohydrate, or lipid
molecule) on a cell surface via the new cysteine in the
β2-M subunit. To accomplish this, the surface of the
cell can be reduced in mild conditions with a reducing
reagent (e.g., dithiothreitol ("DDT"), 2-mercaptoethanol,
10 or 2-mercaptoethylamine·HCl), resulting in reactive sites
(e.g., sulfhydryl groups) on the cell surface. Such
reactive sites will then be reacted directly, or linked
through a bifunctional crosslinker, with the free
sulfhydryl group in the class I molecule. By way of
15 example, the sulfonyl groups attached to the succinimidyl
rings in Sulfo-NHS esters (e.g., Sulfo-LC-SPDP or Sulfo-
SMCC) make these crosslinkers membrane-impermeable and
thus non-reactive with inner membrane proteins; thus,
these crosslinkers are useful in crosslinking the new
20 class I complexes to the cell surface. To determine
optimal conjugation conditions, class I negative cells
such as human HMy2.C1R cells (American Type Culture
Collection CRL-1993) can be used. The density of the
class I peptide complexes anchored on the cell surface
25 can be determined by fluorescence-activating cell sorting
("FACS") analysis, using monoclonal antibodies ("MAb"s)
against the class I molecule.

Peptide-class I complexes conjugated to syngeneic
cells can be used to stimulate the immunity in an
30 individual. To do this, cells derived from this
individual or an another with matching MHC haplotypes are
conjugated *in vitro* to the new class I molecules that
have been loaded with antigenic peptides of interest.
Useful cells include, but are not limited to, peripheral

- 10 -

blood lymphocytes taken from a Ficoll density gradient, purified antigen presenting cells such as macrophages/monocytes, dendritic cells, and B cells, or red blood cells. Antigenic peptides of interest are, for
5 example, melanoma-associated immunodominant epitopes derived from melanoma-associated antigens such as MART-1/Melan A, gp 100/Pmel 17, tyrosinase, Mage 3, p15, TRP-1, and β-catenin (Tsomides et al., *International Immunol.*, 9:327-338). The cell conjugates are then
10 introduced into the individual. If the conjugates are used for vaccination, it may be preferred to use antigen-presenting cells as the conjugates' cellular components, since these cells can provide the requisite costimulatory signals for inducing an effective T cell response.
15   The above immunization strategy allows the control of epitope density and circumvents a variety of problems associated with classical vaccination strategies. For instance, unlike traditional peptide vaccines, the peptides embedded in the present pre-formed, class I
20 peptide complexes are protected from rapid enzymatic degradation or intracellular processing. Traditional peptide vaccines are generally not directly presented by class I molecules on the cell surface; instead, they are typically internalized and processed inside the cell for
25 association with MHC class I molecules. Peptide presentation is thus dependent on a series of intracellular events including the rate of protein degradation, peptide transport, and competition with endogenous peptides.

30 EXAMPLE 1
     Direct Conjugation of Class I Complexes with Cells
           10^6 cells in PBS are reduced with 50 μM DDT for 30 minutes. Consequently, reactive -SH groups on the cell surface are generated. After two washes with PBS, the
35 reduced cells are incubated with the new class I

- 11 -

complexes, resulting in formation of disulfide bonds
between the -SH groups in the β2-M subunits and the -SH
groups on the surface of the reduced cells.

EXAMPLE 2
5 Indirect Conjugation of Class I Molecules with Cells
10^6 cells are suspended in 500 μl PBS buffer (pH
7.2), and 1 mg of Sulfo-SMCC is added to the cell
solution. The incubation proceeds for an hour at room
temperature or 30 minutes at 37°C, resulting in a covalent
10 bond between the NHS-ester group in Sulfo-SMCC and a
primary amine on the cell surface.
The cells are then washed three times with PBS,
and incubated with 0.5 mg of the new recombinant class I
protein at 4°C for 1 hour. This step leads to formation
15 of a covalent bond between the free -SH group in the new
class I protein and the maleimide group in Sulfo-SMCC.

II.   Antibody-Class I Conjugates
The new MHC class I molecule can be specifically
targeted to a cell by conjugating to an antibody against
20 an antigen expressed on the surface of the cell. For
instance, non-self (e.g., allogeneic) class I molecules
conjugated to antibodies specific for a tumor antigen (or
any antigen exclusively or primarily expressed by any
other undesired tissue) can be attached to tumor tissue
25 (or the undesired tissue) in an individual. The tissue,
which now bears foreign MHC molecules, becomes a target
of allograft rejection, one of the strongest immune
responses known, and can thereby be destroyed.
To elicit a strong immune response against
30 undesired tissue in an individual, an antibody specific
for the tissue can also be conjugated to a syngeneic MHC
class I molecule that is associated with a potent T cell
epitope (e.g., HLA-A2 with the influenza matrix protein
59-68 (ILGFVFTLTV, SEQ ID NO:2), or the influenza B NP

- 12 -

85-94 (KLGEFYNQMM; SEQ ID NO:3)). Such a conjugate can
elicit a strong zcytotoxic T cell response that
eradicates the undesired tissue.

A variety of monoclonal antibodies can be used to
5 target tumor tissue. Exemplary tumor-associated antigens
include, but are not limited to, the Lewis^y-related
carbohydrate (found on epithelial carcinomas), the IL-2
receptor p55 subunit (expressed on leukemia and lymphoma
cells), the erbB2/p185 carcinoma-related proto-oncogene
10 (overexpressed in breast cancer), gangliosides (e.g.,
GM2, GD2, and GD3), epithelial tumor mucin (i.e., MUC-1),
carcinoembryonic antigen, ovarian carcinoma antigen
MOv-18, squamous carcinoma antigen 17-1A, and malignant
melanoma antigen MAGE. To extend the serum half life of
15 the MHC-antibody conjugate, the antibody may be humanized
if the tumor to be treated is in a human.

The antibody and the MHC components in the present
conjugate are conjugated via a covalent bond between the
new cysteine residue in the mutant β2-M and a functional
20 group in the antibody. Intermediary crosslinkers can be
used to form the covalent bond. For example, mild
oxidation of the sugar moieties in an antibody, using,
e.g., sodium metaperiodate, will convert vicinal
hydroxyls to aldehydes or ketones. The reaction will be
25 restricted to sialic acid residues when 1 mM sodium
metaperiodate is used at 0° C. Subsequent reaction of
the aldehyde or ketone group with a sulfhydryl-reactive,
hydrazide-containing crosslinker (e.g., 3-(2-
Pyridyldithiol)propionyl hydrazide ("PDPH"), 4-(N-
30 maleimidomethyl)cyclohexane-1-carboxylhydrazide
hydrochloride, or 4-(4-N-maleimidophenyl)butyric acid
hydrazide hydrochloride) results in the formation of a
hydrazone bond. The antibody can then be bonded to the
new MHC class I molecule via a disulfide bond formed

- 13 -

between the new cysteine residue in the β2-M and the
sulfhydryl-reactive group in the crosslinker.

   Alternatively, mild reduction of an immunoglobulin
can generate free sulfhydryl groups from the disulfide
5 bonds in the hinge region. The sulfhydryl groups can
then be reacted with the free sulfhydryl group of the
class I complex, either directly or via a
homobifunctional crosslinker.

   Antibody fragments (e.g., Fab, or F(ab)'$_2$, both of
10 which lack the glycosylated Fc-portion) can also be
conjugated to the class I complex. F(ab)'$_2$ fragments can
be generated from antibody molecules by pepsin cleavage;
these fragments can be linked through heterofunctional
cross-linkers such as those that are amine- and
15 sulfhydryl- reactive. Reduction of F(ab)'$_2$ fragments
generates Fab' fragments, which contain free sulfhydryl
groups. These free sulfhydryl groups can be utilized in
conjugation with the new class I proteins.

Example
20 Generation of an Antibody-Class I Conjugate
   The following is an exemplary protocol for
conjugating an antibody to the new MHC class I molecule.
   An immunoglobulin G ("IgG") stock solution is
first prepared. It contains 2mg/ml IgG (i.e., 13 μM) in
25 0.1 M sodium acetate buffer (pH 5.5). The solution is
stored at 0°C. To oxidize the antibody, 1 ml of the IgG
stock solution is mixed with 0.1 ml of cold sodium
metaperiodate solution (stock: 1 mM sodium periodate in
0.1 M sodium acetate buffer, pH 5.5) for 20 min at 0°C in
30 the dark. To stop the oxidation reaction, glycerol is
added to reach a final concentration of 15 mM, and the
incubation proceeds for 5 additional minutes at 0°C.
   The IgG sample is then dialyzed over night against
0.1 M sodium acetate buffer (pH 5.5) and concentrated in
35 a CENTRICON 30. The crosslinker PDPH is then added to

- 14 -

the sample to a final concentration of 5 mM, and the
sample is incubated for approximately 2 hours at room
temperature.

The IgG protein attached to PDPH is purified by
5 HPLC (i.e., high performance liquid chromatography) gel
filtration. The HPLC running buffer is 0.1 M Tris-Cl
(pH 8). The IgG peak fraction is concentrated with
CENTRICON 30 to a final volume of 100 μl.

To prepare for the class I molecule in the MHC-
10 antibody conjugate, 2 mg of HPLC-purified class I
molecule is mildly reduced with a solution containing 0.1
mM DTT and 0.1 M Tris-Cl (pH 8) for 1 hour at room
temperature. This will maintain the cysteine in a
reduced state. The class I molecule preparation is then
15 purified by HPLC, which removes DTT, and concentrated
with CENTRICON 30.

2 mg of the resulting class I molecule is
dissolved in 0.5 ml Tris-Cl (pH 8), and added to 0.5 ml
of PDPH-modified IgG for overnight incubation at 4°C.
20 The sample is then concentrated with CENTRICON 100 and
the MHC-antibody conjugate so obtained, which is
approximately 200 kD in size, is purified by HPLC gel
filtration or fast protein liquid chromatography. The
conjugate is concentrated with CENTRICON 100. Excess
25 peptide (e.g., 5-15 fold molar excess) is added to the
concentrated conjugate preparation to stabilize the class
I molecule in the conjugate.

III. Multimeric MHC Class I Complexes

The new MHC class I molecule can also be used to
30 form a multimeric MHC class I complex. To do so, one can
first obtain a conjugate of a monomeric class I molecule
and a ligand for a multivalent binding molecule. The
conjugate is formed specifically via a linkage between
the sulfhydryl group of the new cysteine in the $\beta 2$-M

subunit and a functional group of the ligand. Useful
ligands include, but are not limited to, iodoacetyl-LC-
biotin, N-(6-[Biotinamido] hexyl)-3'-(2'pyridyldithio)-
propionamide ("biotin-HPDP"), and 1-Biotinamido-4-(4'-

5   [maleimidomethyl] cyclohexane-carboxamido)butane
("Biotin-BMCC"). All these biotin derivatives bind to
avidin (or a derivative thereof such as streptavidin), a
tetravalent molecule. Quantitative blocking of the
biotin-binding sites on avidin will render the avidin

10 molecule mono-, bi-, or tri-valent. To generate the
conjugate, the α and β2-M subunits may be allowed to
associate in the presence of a peptide of interest to
form a stable heteroduplex complex, and then be linked to
the ligand; alternatively, the β2-M subunit may be—first

15 linked to the ligand, and then allowed to associate with
the α chain in the presence of the peptide. Multimers of
these conjugates can be formed by supplying to these
conjugates the multivalent binding molecule to which the
ligand binds.

20   The multimeric MHC class I complexes can be used
for labeling, quantitation, isolation, and stimulation of
T cells. By way of example, the class I multimers can be
immobilized on solid-phase matrices, generating an
affinity support for the enrichment and isolation of T

25 cells harboring the corresponding antigen receptors. The
matrices may be, for instance, agarose gel, beaded
polymers, polystyrene plates, glass slides,
nitrocellulose membrane, or columns. Immobilization can
be effected by non-covalent coupling (e.g., between

30 biotin-avidin/streptavidin interaction, or through
magnetic field), or covalent coupling. Certain
reversible coupling can allow the captured cells to be
eluded from the affinity support. For instance, if the
complex is biotinylated with Biotin HPDP, the biotin

- 16 -

moiety can be cleaved from the reacted sulfhydryl with a
reducing reagent (e.g., DDT, or β-mercaptoethanol).

The new class I multimers can also be used to
characterize T cells that recognize non-classical class I
5   proteins. This is because the new β2-M can assemble with
a wide variety of class I heavy chains, and thus a wide
variety of class I multimer probes can be generated using
the new β2-M.

The HLA class I gene family (see, e.g., "Phylogeny
10 of the Major Histocompatibility Complex," in
Immunological Reviews, 113:1-241, 1990) includes the
highly polymorphic class I genes HLA-A, HLA-B and HLA-C,
all of which show widespread tissue expression. At least
three additional class I genes HLA-E, HLA-F and HLA-G
15 (known as non-classical class I b genes) have been
identified; these genes are highly homologous to the
classical HLA class I genes, and their polypeptide
products are all associated with β2-M. The family of
non-classical class I genes also includes members that do
20 not reside in the MHC genomic complex, such as the
subfamily of CD1 genes in both mouse and human, and the
mouse thymus leukemia (TL) antigen. Both the TL antigen
and CD1 proteins have the typical structure of an
antigen-presenting class I molecule. They form
25 heterodimers at the cell surface in which a heavy chain
of approximately 38-50 kDa interacts with β2-M. Another
notable non-classical class I gene in mice is the Qa-2
gene. The Qa-2 products, like other class I heavy
chains, are associated with β2-M. The function of these
30 non-classical molecules and the responding T cells are
largely unknown.

The ability of β2-M to associate with diverse
heavy chains, and the use of these complexes as signal
(e.g., fluorescence)-producing probes, can facilitate the
35 characterization of the T cells that react with these

- 17 -

class I molecules.   Information about the corresponding T
cells may provide important clues about their specialized
function in the immune system.

5   Monomeric class I complexes conjugated to a solid
support are in effect multimerized due to their physical
proximity.   Thus, the new, monomeric class I complexes
conjugated to a solid surface via their free -SH groups
can also be used for the same purposes as those described
above for multimeric class I complexes.

10   The following examples describe the generation and
some uses of several new class I MHC-peptide tetramers.
The examples serve to illustrate, but not limit, the new
methods and reagents.

          In these examples, CTL clones from asymptomatic
15 HIV-1 seropositive patients were established and
maintained as described (Johnson et al., *J. Immunol*,
<u>147</u>:1512-1521, 1991).   HLA-typing was performed by the
Massachusetts General Hospital Tissue Typing Laboratory
using standard serological techniques.   H-2K^b-SV9 specific
20 CTL clones (syngeneic reactive) were generated in H-2^b
mice (K^{b}D^b) as described (Zhou et al., *J. Immunol Methods*,
<u>153</u>:193-200, 1992).   The alloreactive CTL were derived
from H-2^{dm2} mice (K^{d}D^d).   All CTLs were maintained in
culture by periodic stimulation with RMA-S cells (H-2^b;
25 see, e.g., Townsend et al., *Cold Spring Harb. Symp.*
Quant. Biol., <u>54 Pt 1</u>:299-308,1989) loaded with SV9
peptide.   CTL clone 4G3, which arose in an H-2K^b mouse,
reacts specifically with the ovalbumin octapeptide pOVA
(SIINFEKL; SEQ ID NO:__) in association with H-2K^b (Walden
30 et al., *Proc. Natl. Acad. Sci. USA*, <u>87</u>:9015-9019, 1990).
Cell surface MHC class I molecules on RMA-S cells are
largely devoid of peptide unless loaded with peptides
from the external medium (Heemels et al., *Ann. Rev.*
Biochem., <u>64</u>:463-491 1995).

- 18 -

EXAMPLE 1
Production of a Mutant β2-M

        The ribosome-binding site and coding region from a
β2-M expression plasmid (Garboczi et al., *Proc. Natl.*
5 *Acad. Sci. USA*, 89:3429-2433, 1992) was cloned into the
E. coli expression vector pLM1 (Garboczi et al., *J.*
Immunol., 157:5403-5410, 1996) to achieve a higher
protein yield. The β2-M polypeptide is of human origin
and has 99 amino acid residues (SEQ ID NO:1) plus one
10 additional methionine residue at the N-terminus that
results from the expression in *E. coli*.

        A cysteine residue was substituted for the
tyrosine 67 residue in the polypeptide by using overlap
extension PCRs (i.e., polymerase chain reactions). Four
15 PCR primers were used. The first two were used at the 5'
and 3' ends of the β2-M coding region in the pHN1 plasmid
(Garboczi et al., *Proc. Natl. Acad. Sci. USA*, 89:3429-
3433, 1992). These two primers were the 5' primer
CTAGAGGATCCTCACACAGGAAACAGAATTTCGAG (SEQ ID NO:4), and
20 the 3' primer CCACCGCGCTACTGCCGCCAGGC (SEQ ID NO:5). The
5' primer introduced a new BamHI site at the 5' end of
the β2-M coding region. The other two primers were used
for mutagenesis. They were the top strand primer CTC TTG
TAC <u>TGC</u> ACT GAA TTC ACC CCC (SEQ ID NO:6), and the bottom
25 strand primer GAA TTC AGT <u>GCA</u> GTA CAA GAG ATA GAA (SEQ ID
NO:7). The new cysteine codon and its complement are
underlined.

        To generate the cysteine mutation, the following
two pairs of primers were first used on the pHN1 template
30 to yield two PCR products: (i) the 5' primer and the
bottom strand primer, and (ii) the 3' primer and the top
strand primer. The two PCR products were electrophoresed
and purified from agarose gel. They were then subjected
together to a PCR reaction using the 5' and 3' primers.
35 The final PCR product, which contains the β2-M coding
region with the cysteine mutation, was digested with

BamHI and HindIII and cloned into the same sites in pLM1.
The mutated β2-M (i.e., "β2-M(Cys67)") sequence was
confirmed by DNA sequencing. Plasmid pLM1 bearing the
β2-M(Cys67)-coding sequence was transformed into *E. coli*
5   host BL21(DE3)plysS (Studier et al., *Methods Enzymol.*,
185:60-89,1990), and a large amount of β2-M(Cys67) was
obtained in the form of inclusion body protein.

EXAMPLE 2
Generation of Peptide-MHC Tetramer
10   To generate a class I MHC-peptide tetramer, the
tyrosine residue at position 67 (SEQ ID NO:1) of human
β2-M was first replaced with a cysteine residue, using
standard mutagenesis techniques (see above). There are
two naturally occurring cysteine residues in β2-M, which
15   maintain the immunoglobulin structure of β2-M by forming
a di-sulfide bond. The new cysteine residue did not bond
with any one of the natural cysteine residues, allowing
the proper folding of the mutant β2-M ("β2-M(Cys67)").
The free sulfhydryl group in the new cysteine residue was
20   to be used for subsequent chemical modifications of the
β2-M subunit.
            A monomeric mutant HLA-A2 containing β2-M(Cys67)
was then generated. The HLA-A2 heavy chain and the
mutant β2-M subunit was obtained in large amounts from *E.*
25   *coli* host cells transformed with the respective
expression vectors. The formation of HLA-A2 (subtype
A0201) was initiated in a dilute solution containing a
denatured HLA-A2 heavy chain (1 μM), the denatured
β_2-M(Cys67) polypeptide (2 μM), and a synthetic peptide
30   (10 μM) (see, e.g., Garboczi et al., *Proc. Natl. Acad.*
Sci. USA, 89:3429-3433, 1992). The folded MHC-peptide
complexes were purified by HPLC (i.e., high performance
liquid chromatography) gel filtration. The protein
concentration of the purified complex was determined by
35   measuring its optical density. For HLA-A2, 1 A_{280} unit

- 20 -

represents 0.67 mg ml^{-1} of the protein. The solvent-
accessible cysteine 67 in the mutant β2-M subunit was
maintained in a reduced state by 0.1 mM DDT.

5   The monomeric mutant HLA-A2 was then biotinylated
with iodoacetyl-LC-biotin ("ILB"; Pierce), a sulfhydryl-
reactive reagent, at the cysteine 67 residue of
β2-M(Cys67). ILB, which was dissolved in N,N-dimethyl-
formamide, was applied at a 5-fold molar excess over the
total amount of cysteine in solution. The biotinylation
10   reaction was carried out in Tris-HCl (pH 8.0) and 0.1 mM
DDT, and the reaction lasted for 1 hour in the dark at
room temperature. Subsequent to the reaction, the volume
of the reaction mix was reduced with CENTRICON 30 from
0.5-1 ml to 50 μl, diluted again in 1 ml Tris-HCl (pH
15   8.0), concentrated to about 100 μl, and purified by HPLC
gel filtration. In the biotinylated β2-M(Cys67), the
biotin moiety of ILB was separated from the sulfhydryl
group of the cysteine 67 residue by the long iodoacetyl-
LC arm.

20   The biotinylated MHC-peptide complexes were
purified by HPLC gel filtration. The purified complexes
were then multimerized in the presence of deglycosylated
avidin-phycoerythrin ("avidin-PE"; Molecular Probes).
Deglycosylated avidin-PE binds more than 12 μg of biotin
25   per mg protein. The resulting tetrameric MHC-peptide
complexes were subjected to HPLC gel filtration (TSK G
3000SW, TOSO HAAS, Gel Filtration Standard/BIO-RAD) and
then concentrated (CENTRICON 100, Amicon).

EXAMPLE 3
30   Labeling of T cells with a Peptide-Class I Tetramer
        A peptide-MHC class I tetramer was prepared with a
HIV-1 gag peptide (i.e., SL9, whose sequence is SLYNTVATL
(SEQ ID NO:8)) (Johnson et al., *J. Immunol*, 147:1512-
1521, 1991), and a control peptide from HIV reverse
35   transcriptase (i.e., IV9, whose sequence is ILKEPVHGV

- 21 -

(SEQ ID NO:9)) (Tsomides et al., *Proc. Natl. Acad. Sci. USA*, 88:11276-11280 1991). The tetramer was formed by use of avidin-PE, as described in the preceding Example. The binding of the tetramer to cytotoxic T lymphocyte
5 ("CTL") clones with known antigen-binding specificity was monitored by flow cytometry.

Specifically, 5×10^5 cells from each of three human CTL clones specific for HIV-1 derived peptides were pre-stained with an anti-CD8 monoclonal antibody ("mAb") Cy-
10 Chrome (Pharmingen). These three clones, i.e., 68A62 (Tsomides et al., *Proc. Natl. Acad. Sci. USA*, 88:11276-11280, 1991), 18030 (Johnson et al., *J. Immunol.*, 147:1512-1521, 1991), 63D35 are specific for HLA-A2-IV9 (i.e., IV9 presented by HLA-A2), HLA-A2-SL9, and HLA-B11-
15 AK9 (AK9: a HIV-1 reverse transcriptase peptide with the sequence of AIFQSSMTK (SEQ ID NO:10)), respectively.

The pre-stained cells were then incubated with the soluble, PE-labeled tetrameric HLA-A2-SL9 complex at a concentration of 50 µg/100 µl RPMI at 4°C for an hour.
20 The cells were washed and subjected to FACS (i.e., fluorescence-activating cell sorting) analysis using a FACSCAN flow cytometer (Becton Dickinson).

In the FACS analysis, contour plots were based on 10,000 events gated on forward- versus side-scatter. An
25 arbitrarily set boundary on the PE-fluorescence intensity of a negative control was the position of the X-axis quadrant marker, implying that about 80% of the cells can be considered as positive for PE-fluorescence.

The data showed that there was a 10-fold increase
30 in the PE fluorescence intensity when the PE-labeled tetramer stained CTLs of clone 18030 (Fig. 1B), as compared to CTLs of clones 68A82 and 63D35 (Figs. 1A and 1C). Thus, the staining of CTLs by the HLA-A2-SL9 tetramer was specific.

- 22 -

A similar shift of PE fluorescence intensity was observed with CTLs of clone 68A62 when those CTLs were stained with a HLA-A2-IV9 tetramer.

Based on quadrant markers, about 80% of the CTLs
5 specific for HLA-A2-SL9 were stained positively with the phycoerythrin-labeled tetramer.

EXAMPLE 4
Labeling of T Cells with a Hybrid Peptide-Class I Complex

The mutant human β_2-M in which Tyr 67 has been
10 replaced with a cysteine residue was also shown to bind stably to the heavy chains of the mouse MHC class I molecule K^b.

Tetrameric MHC class I molecules were generated with use of a purified murine H-2K^b ("K^b") heavy chain
15 (Zhang et al., *Proc. Natl. Acad. Sci. USA*, <u>89</u>:8403-8407, 1992), the mutant human β2-M, and SV9, a Sendai virus nucleoprotein-derived peptide (FAPGNYPAL, SEQ ID NO:11) (Kast et al., *Proc. Natl. Acad. Sci. USA*, <u>88</u>:2283-2287, 1991; and Schumacher et al., *Nature*, <u>350</u>:703-706, 1991).
20 SV9 promoted folding of the hybrid K^b molecule with an efficiency similar to that of SL9 for promoting folding of HLA-A2. The purified K^b tetramer migrated as a single peak on gel filtration HPLC. The presence of the heavy chain and the β_2-M subunit in the peak fractions was
25 confirmed by SDS polyacrylamide gel electrophoresis.

5x10^5 murine CTLs of three different clones were pre-stained with anti-CD8 TRI-COLOR (CALTAG), and then incubated with 50 μg/100 μl RPMI PE-labeled chimeric H-2K^b-SV9 tetramer for an hour at 4°C. The three CTL clones
30 2F3, 3C2, and 4G3 (Walden et al., *Proc. Natl. Acad. Sci. USA*, <u>87</u>:9015-9019,1990) are specific for SV9 bound to H-2K^b, SV9 bound to H-2D^b, and OVA (SIINFEKL, SEQ ID NO:12); derived from ovalbumin; Walden et al., *Proc. Natl. Acad. Sci. USA*, <u>87</u>:9015-9019) bound to H-2K^b, respectively.

- 23 -

(The specificity of the clones is determined on the basis
of their lysis of target cells loaded with the
corresponding peptide.) After the incubation, the cells
were washed and subjected to FACS analysis.

5   Histogram plots (Figs. 2A-2C) for the FACS
analysis were based on 10,000 events gated on CD8
fluorescence. As shown in the plots, the K^b-SV9 tetramer
stained only 2F3 CTLs (Fig. 2A), but not 3C2 (Fig. 2B) or
4G3 (Fig. 2C) CTLs.

10   In the FACS analysis, markers (M1, M2) were placed
to delineate a region of positive intensity relative to a
control (Figs. 2B and 2C). Based on these markers, 78%
of 2F3 CTLs (Fig. 2A) were found to stain positive with
the K^b-SV9 tetramer.

15 EXAMPLE 5
    Binding of T Cells by Allogeneic Peptide-Class I
    Complexes
         Allograft rejection is probably the most powerful
    T cell reaction known. During such a rejection, an
20 individual's T cells respond strongly to target cells
    (e.g., a skin graft from a genetically disparate
    individual of the same species) that bear allogeneic MHC
    molecules. It has been suggested that the affinities of
    T cell receptors for MHC-peptide complexes be higher when
25 the MHC component is allogeneic (i.e., non-self) than
    syngeneic (i.e., self) Sykulev et al., *Proc. Natl. Acad.
    Sci. USA*, 91:11487-11491, 1994 and Eisen et al., *Advances
    in Protein Chemistry*, 49:1-56, 1996).
         Figs. 3A-3D compare the reactivity of the K^b-SV9
30 tetramer with murine CD8+ CTLs from two different mouse
    lines. Briefly, 5x10^5 syngeneic MHC- and allogeneic MHC-
    CTLs were stained with anti-CD8 TRI-COLOR and anti-H-2K^b-
    FITC mAbs (CALTAG), washed and FACS analysed. The CTLs
    were then incubated with the H-2K^b SV9 tetramer-PE reagent
35 (50 μg/100 μl RPMI, 4°C, 1h), washed, and again subjected

- 24 -

to FACS analysis. All three fluorochromes were excited
with an argon laser in a single laser instrument (total
of 10,000 events collected).

        The CTLs in Fig. 3A were derived from a
5   K^b-positive mouse and the reaction shown is syngeneic;
whereas in Fig. 3C, the CTLs were derived from a K^b-
negative mouse and the reaction shown is allogeneic. The
tetramer bound specifically to CTLs from both mouse lines
(Figs. 3B and 3D), but it distinguished between two
10 subsets in the alloreactive CTLs (Fig. 3D), the better
binding subset amounting to about 12-15% of the total CTL
population. The observed difference in staining
intensities of CTLs (Fig. 3D) might be influenced by
differences in cell surface density of TCR and perhaps
15 CD8 molecules. Assuming a 1:1 ratio of the TCR
heterodimer with the CD3 complex, CD3 and CD8 expression
levels were analyzed. The analysis showed that the
cellular subsets of the allogeneic CTL line that stained
with multimeric K^b-SV9 of different intensities had the
20 same amount of CD8 and CD3 per cell.

                      Other Embodiments
        It is to be understood that while the invention
has been described in conjunction with the detailed
description thereof, the foregoing description is
25 intended to illustrate and not limit the scope of the
invention, which is defined by the scope of the appended
claims. For example, to attach a foreign class I
molecule (e.g., one with an allogeneic or even xenogeneic
heavy chain) to a tumor cell, a ligand for a receptor
30 specifically expressed on the tumor cell, instead of a
tumor-specific antibody, is conjugated to the foreign MHC
class I molecule to direct an alloresponse to the tumor
tissue.

Other aspects, advantages, and modifications are within the scope of the following claims.

- 26 -

What is claimed is:

1. A method of preparing a conjugate of a MHC class I molecule and a compound, the method comprising:
    providing a MHC class I molecule, wherein a

5   cysteine residue is introduced into its β2-microglobulin subunit; and
    conjugating the MHC class I molecule and the compound, specifically via a linkage formed between the sulfhydryl group of the cysteine residue in the

10 β2-microglobulin subunit and a functional group of the compound.

2. The method of claim 1, wherein the cysteine residue is introduced into a region corresponding to residues 15-23, 35-53, or 66-97 of SEQ ID NO:1.

15   3. The method of claim 2, wherein the cysteine residue is a substitute for a residue corresponding to position 67 of SEQ ID NO:1.

4. The method of claim 1, wherein the compound is a ligand for a multivalent binding molecule.

20   5. The method of claim 4, wherein the cysteine residue is a substitute for a residue corresponding to position 67 of SEQ ID NO:1.

6. The method of claim 1, wherein the compound is an antibody.

25   7. The method of claim 6, wherein the antibody is specific for a tumor antigen.

- 27 -

8.   The method of claim 7, wherein the cysteine residue is a substitute for a residue corresponding to position 67 of SEQ ID NO:1.

9.   The method of claim 1, wherein the compound is on the surface of a cell.

10.   The method of claim 9, wherein the compound is a protein.

11.   The method of claim 9, wherein the cell is an antigen-presenting cell.

12.   The method of claim 11, wherein the cysteine residue is a substitute for a residue corresponding to position 67 of SEQ ID NO:1.

13.   The method of claim 1, wherein the compound is a ligand for a surface receptor of a cell.

14.   The method of claim 13, wherein the cell is an antigen-presenting cell.

15.   The method of claim 14, wherein the cysteine residue is a substitute for a residue corresponding to position 67 of SEQ ID NO:1.

16.   A method of making a multimeric MHC class I molecule, the method comprising:
        providing a conjugate of a monomeric MHC class I molecule and a ligand for a multivalent binding molecule, wherein a cysteine residue is introduced into the $\beta 2$-microglobulin subunit of the MHC class I molecule, the conjugate is formed specifically via a linkage between the sulfhydryl group of the cysteine residue in the

- 28 -

β2-microglobulin subunit, and a functional group of the
ligand; and

 attaching a plurality of the conjugates, via the
ligand, to the multivalent binding molecule to form a
5 multimeric MHC class I molecule.

 17. The method of claim 16, wherein the cysteine
residue is introduced into a region corresponding to
residues 15-23, 35-53, or 66-97 of SEQ ID NO:1.

 18. The method of claim 17, wherein the cysteine
10 residue is a substitute for a residue corresponding to
position 67 of SEQ ID NO:1.

 19. The method of claim 16, wherein the ligand is
biotin, and the multivalent binding molecule is
streptavidin or avidin.

15 20. A method of making a multimeric MHC class I
molecule, the method comprising:

 providing a β2-microglobulin into which a cysteine
residue is introduced;

 conjugating the β2-microglobulin and a ligand for
20 a multivalent binding molecule, specifically via a
linkage formed between the sulfhydryl group of the
cysteine residue in the β2-microglobulin and a functional
group of the ligand;

 mixing the conjugate so obtained with an α chain
25 of an MHC class I molecule to form a monomeric MHC class
I molecule; and

 attaching a plurality of the monomeric MHC class I
molecules so obtained to the multivalent binding
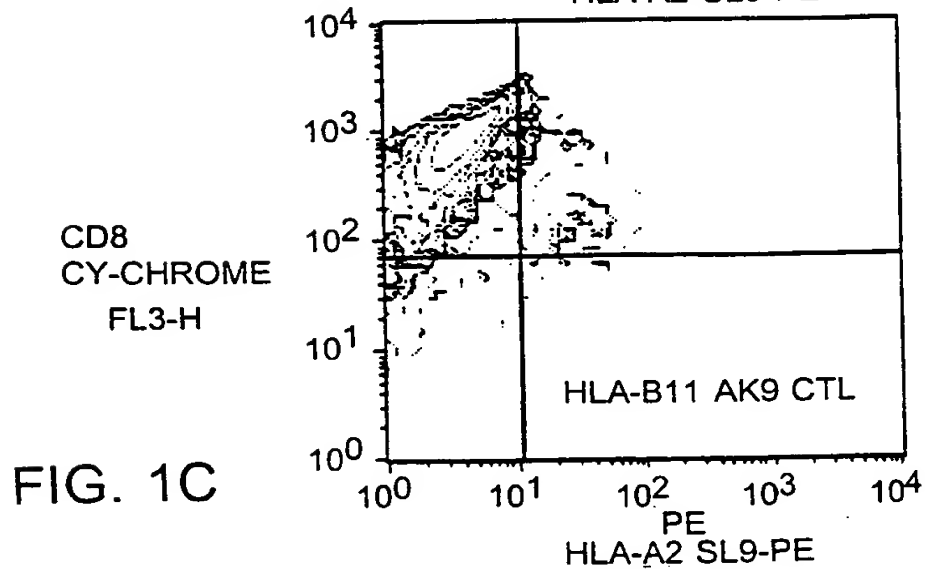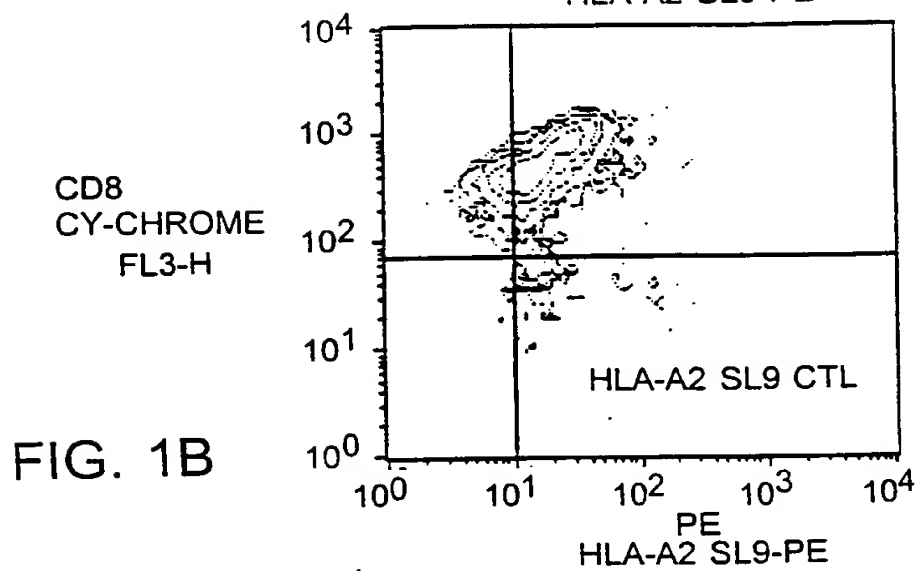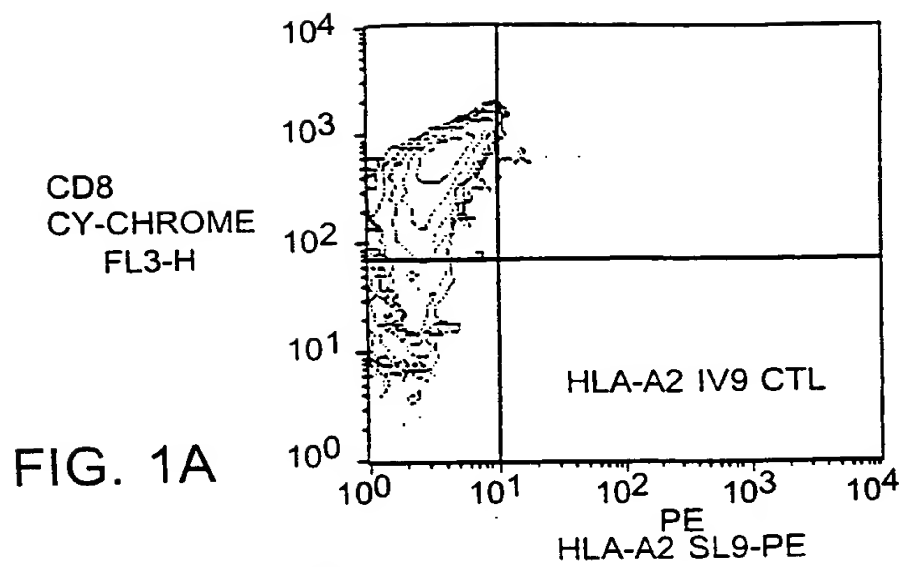molecule, via the ligand, to form a multimeric MHC class
30 I molecule.

- 29 -

21.   The method of claim 20, wherein the cysteine
residue is introduced into a region corresponding to
residues 15-23, 35-53, or 66-97 of SEQ ID NO:1.

22.   The method of claim 21, wherein the cysteine
5 residue is a substitute for a residue corresponding to
position 67 of SEQ ID NO:1.

23.   The method of claim 20, wherein the ligand is
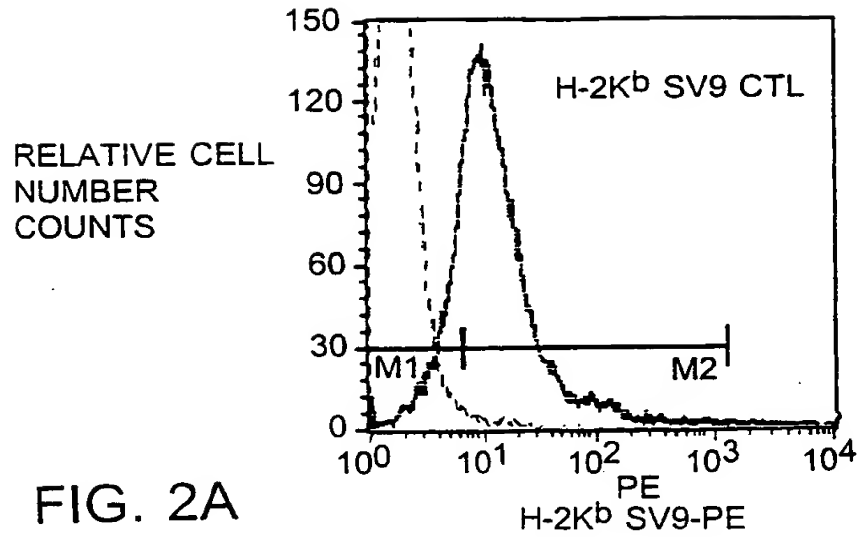biotin, and the multivalent binding molecule is
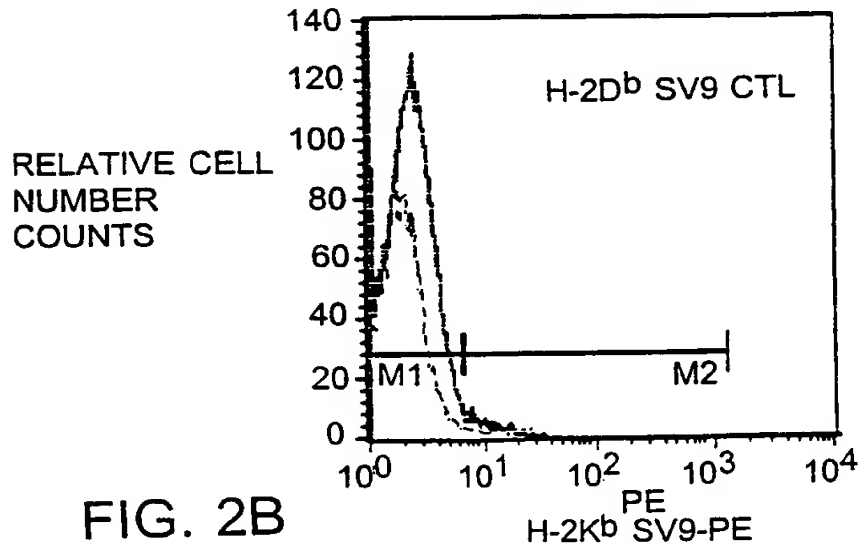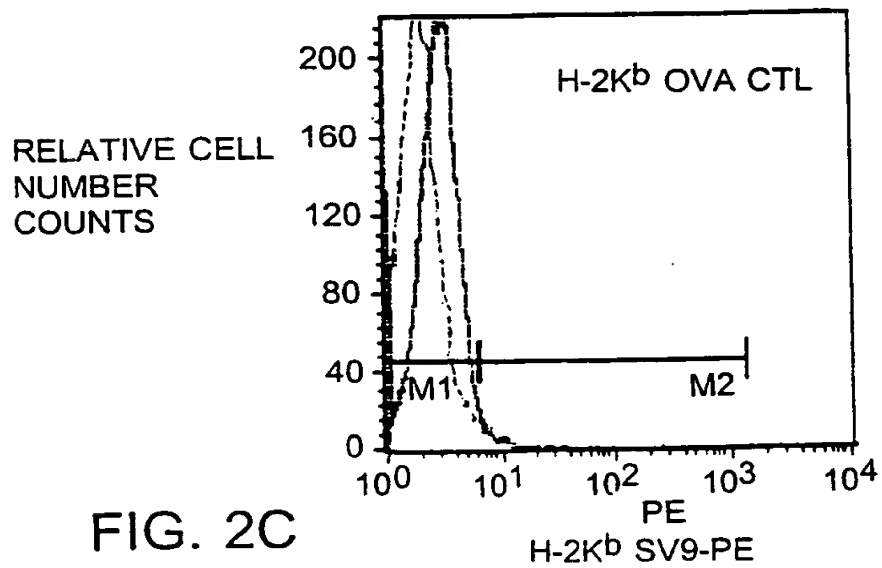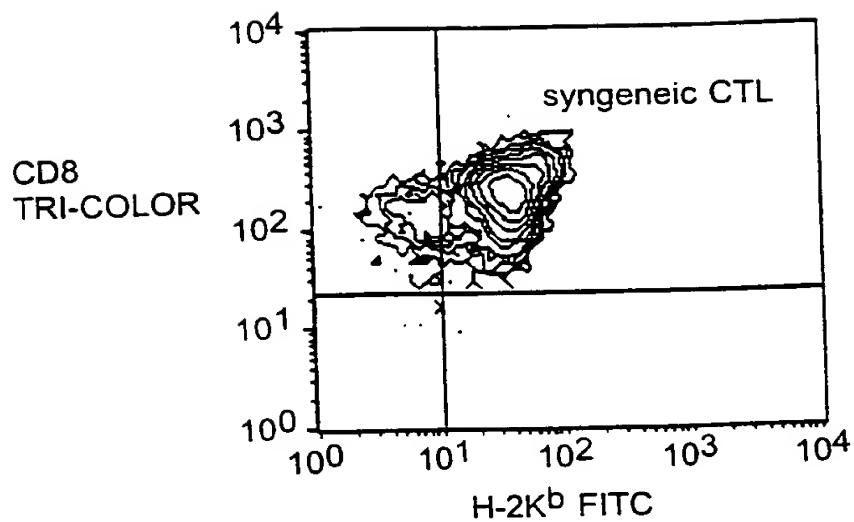streptavidin or avidin.

1/4

FIG. 1A

FIG. 1B

FIG. 1C

RELATIVE CELL
NUMBER
COUNTS

H-2K^b SV9 CTL

FIG. 2A

PE
H-2K^b SV9-PE

RELATIVE CELL
NUMBER
COUNTS

H-2D^b SV9 CTL

FIG. 2B

PE
H-2K^b SV9-PE

RELATIVE CELL
NUMBER
COUNTS

H-2K^b OVA CTL

FIG. 2C

PE
H-2K^b SV9-PE

3/4

FIG. 3A
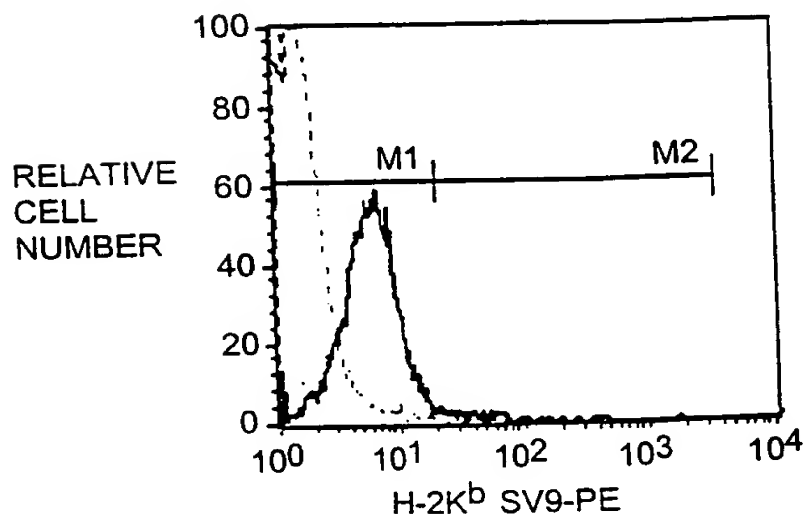
FIG. 3B

4/4
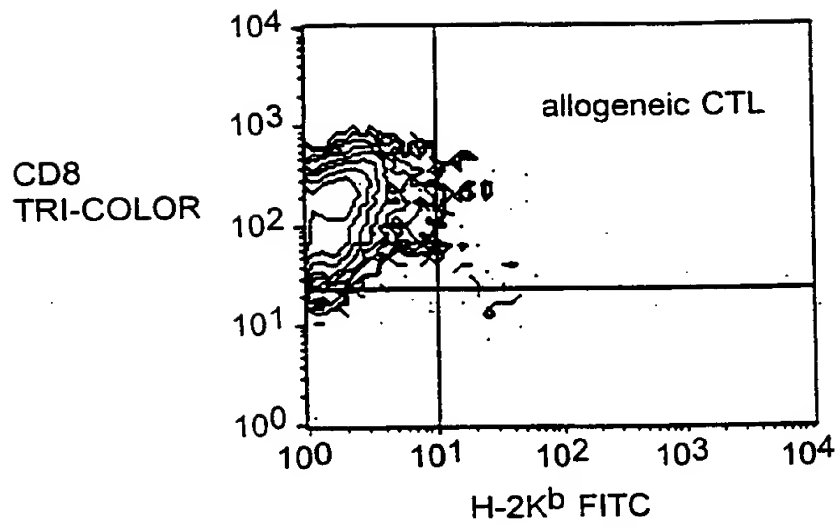
FIG. 3C

FIG. 3D

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/09, 15/12; C07K 14/74
US CL :435/69.1, 69.7; 530/387.3, 395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7; 530/387.3, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System (APS): key words: major histocompat? cysteine, disulfide, conjugate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, 4,400,376 A (SANDERSON) 23 August 1983, see entire document.	1-23
Y	US 4,478,823 A (SANDERSON) 23 October 1984, see entire document.	1-23
Y	US 5,130,297 A (SHARMA ET AL.) 14 July 1992, see entire document.	1-23
Y	WEIR, D.M. et al. (ed), HANDBOOK OF IMMUNOLOGY 4th edition: IMMUNOCHEMISTRY, published 1986, Blackwell Scientific Publications, Oxford, UK. pages 3.1-3.13. See pages 3.1-3.13.	1-23
Y	US 5,153,265 A (SHADLE ET AL.) 06 October 1992, see entire document.	1-23

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 SEPTEMBER 1998	27 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Thomas Cunningham
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,698,420 (URNOVITZ) 06 October 1987, see entire document.	1-23

THIS PAGE BLANK (USPTO)